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Cell-free synthesis and assembly of prolyl 4-hydroxylase: the role of the β-subunit (PDI) in preventing misfolding and aggregation of the α-subunit

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Prolyl 4-hydroxylase (P4-H) catalyses a vital post-translational modification in the biosynthesis of collagen. The enzyme consists of two distinct polypeptides forming an $\alpha_2\beta_2$ tetramer (α = 64 kDa, β = 60 kDa), the β-subunit being identical to the multifunctional enzyme protein disulfide isomerase (PDI). By studying the cell-free synthesis of the rat α-subunit of P4-H we have shown that the α-subunit can be translocated, glycosylated and the signal peptide cleaved by dog pancreatic microsomal membranes to yield both singly and doubly glycosylated forms. When translations were carried out under conditions which prevent disulfide bond formation, the product synthesized formed aggregates which were associated with the immunoglobulin heavy chain binding protein (BiP). Translations carried out under conditions that promote disulfide bond formation yielded a product that was not associated with BiP but formed a complex with the endogenous β-subunit (PDI). Complex formation was detected by co-precipitation of the newly synthesized α-subunit with antibodies raised against PDI, by sucrose gradient centrifugation and by chemical cross-linking. When microsomal vesicles were depleted of PDI, BiP and other soluble endoplasmic reticulum proteins, no complex formation was observed and the α-subunit aggregated even under conditions that promote disulfide bond formation. We have therefore demonstrated that the enzyme P4-H can be assembled at synthesis in a cell-free system and that the solubility of the α-subunit is dependent upon its association with PDI.

Key words: BiP/cell-free synthesis/prolyl 4-hydroxylase/protein disulfide/subunit assembly

Introduction

Prolyl 4-hydroxylase (P4-H; E.C. 1.14.11.2), an enzyme located in the rough endoplasmic reticulum (ER), catalyses the co- and post-translational hydroxylation of proline residues in X-Pro-Gly sequences of collagens and proteins with collagen-like amino acid sequences (recently reviewed by Kivirikko *et al.*, 1992). The presence of 4-hydroxyproline residues in newly synthesized procollagen polypeptide chains is essential for the folding of the chains to form triple-helical molecules that are stable at body temperature (Berg and Prockop, 1973); unhydroxylated or incompletely hydroxylated chains accumulate within the cisternae of the rough ER and are slowly secreted as non-functional protein (Prockop *et al.*, 1976). Inhibition of the hydroxylation reaction therefore prevents triple-helix formation and results in a reduction in the amount of functional collagens secreted from the cell.

Apart from its proline-rich polypeptide substrate, P4-H requires Fe(II), 2-oxoglutarate, O_2 and ascorbate for the hydroxylation reaction in which the 2-oxoglutarate is stoichiometrically decarboxylated; one atom of the O_2 molecule being incorporated into the succinate product while the other is incorporated into the hydroxyl group formed on the proline residue (Kivirikko *et al.*, 1992). Various fibrotic disorders, e.g. liver cirrhosis, are characterized by excessive collagen (extracellular matrix) deposition, consequently there has been considerable interest in P4-H as a potential target for therapeutic modulation of such conditions (Franklin, 1992). Recently, as a result of previous studies detailing the substrates, co-factors and kinetics of the P4-H reaction (Kivirikko *et al.*, 1992) and its possible mechanism (Hanauske-Abel and Gunzler, 1982), liver selective inhibitors of P4-H have been developed (Bickel *et al.*, 1991).

Vertebrate P4-H consists of two distinct polypeptides—the catalytically more important, glycosylated, α-subunit (64 kDa) and the β-subunit (60 kDa) which is identical to the multifunctional enzyme protein disulfide isomerase (PDI; Pihlajaniemi *et al.*, 1987). The enzyme appears to be assembled *in vivo* into an $\alpha_2\beta_2$ tetramer from newly synthesized α-subunit associating with an endogenous pool of β-subunit (Berg *et al.*, 1980). Attempts to reassociate the isolated subunits *in vitro* to form an active enzyme tetramer have been unsuccessful (Nietfeld *et al.*, 1981; Koivu and Myllyla, 1986) due to the aggregation tendency and consequent insolubility of the α-subunit on dissociation from the β-subunit (Tuderman *et al.*, 1975). One of the possible roles of the β-subunit (PDI) may be to maintain the α-subunit in a non-aggregated form, a role proposed for PDI as a component of the microsomal triglyceride transfer protein complex (Wetterau *et al.*, 1991). PDI also possesses a carboxy terminal KDEL sequence which is both necessary and sufficient for retention of a polypeptide within the ER (Munro and Pelham, 1987); association of the α-subunit with the β-subunit may thus serve to retain the enzyme within the ER (Vuori *et al.*, 1992).

In view of the lack of information on the interactions of the α-subunits with the β-subunit in forming the P4-H tetramer, we have developed a cell-free system which reconstructs the initial stages in the synthesis of this enzyme. An RNA transcript coding for the α-subunit was transcribed *in vitro* from a rat cDNA clone and translated in a cell-free system consisting of a rabbit reticulocyte lysate optimized for the formation of disulfide bonds supplemented with canine pancreatic microsomal vesicles. Under these conditions the α-subunit synthesized formed a complex with the β-subunit which prevented its aggregation. Under

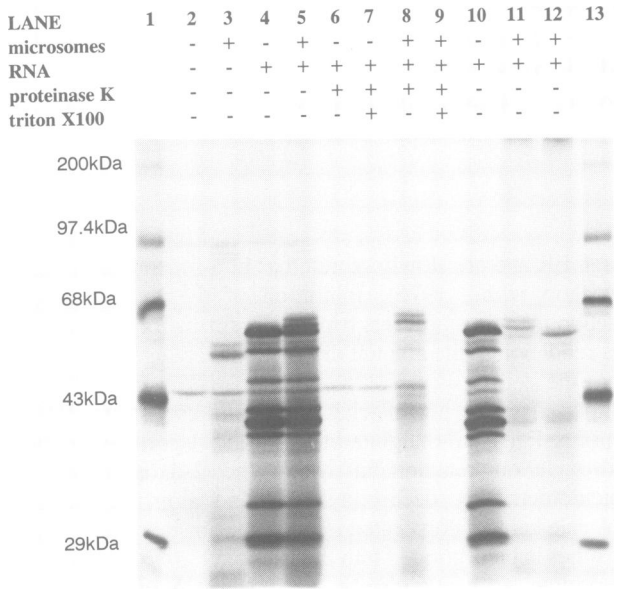

Fig. 1. Cell-free translation, translocation and glycosylation of P4-H α-subunit. P4-H α-subunit RNA was synthesized by *in vitro* transcription of pDJ2 linearized with *SalI* using T3 RNA polymerase. Translation of purified α-subunit RNA was carried out using a rabbit reticulocyte lysate system supplemented with amino acids (minus methionine), [^{35}S]methionine and dog pancreatic rough ER microsomes as required. Translations were carried out at 30°C for 60 min and terminated by incubation on ice. Products of translation were separated by SDS–PAGE through a 12.5% polyacrylamide gel. The gel was fixed, dried and developed by autoradiography. Samples of control translations carried out in the absence of RNA are shown in lanes 2 and 3; all other lanes contain samples derived from translations of α-subunit RNA in the presence or absence of microsomes as indicated. Proteinase K treatment (lanes 6–9) was carried out at 4°C either in the absence (lanes 6 and 8) or presence of Triton X-100 to disrupt the microsomes (lanes 7 and 9). Microsomes isolated by centrifugation of translation mixtures through sucrose cushions were used for EndoH treatment (lanes 11 and 12). Lanes 1 and 13 contained ^{14}C-labelled protein standards of the sizes indicated.

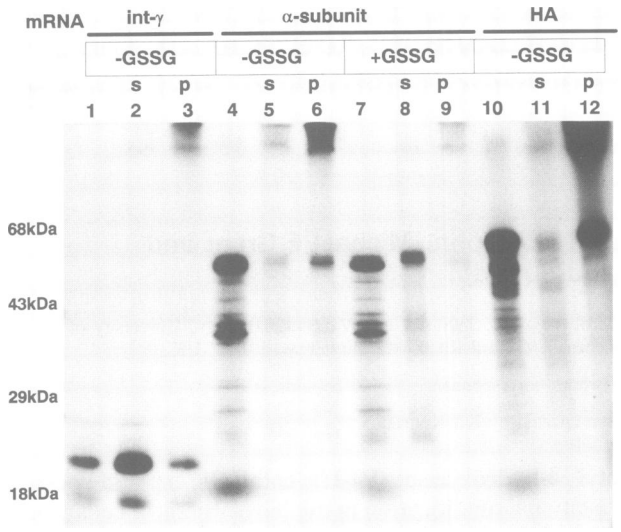

Fig. 2. Membrane association of translation products: sodium carbonate treatment. Microsomes were isolated from translation reactions containing the indicated RNA transcripts (int-γ = interferon γ; α-subunit = P4-H α-subunit; HA = influenza virus haemagglutinin) by centrifugation through sucrose cushions. Products of translation were separated by SDS–PAGE. The gel was fixed, soaked in Amplify, dried and developed by fluorography. GSSG was either included (lanes 7–9) or excluded (lanes 1–6 and 10–12) from translation reactions. Purified microsomes were subjected to sodium carbonate treatment to yield pellet and supernatant fractions. Aliquots of the original translation samples (prior to microsome isolation) are shown in lanes 1, 4, 7 and 10, samples in lanes marked S and P are supernatant and pellet fractions, respectively. The positions of protein standards were as indicated.

conditions where disulfide bonds are unlikely to be formed, the α-subunit aggregated, did not form a complex with the β-subunit but was associated with immunoglobulin heavy chain binding protein (BiP).

Results

Cell-free translation of P4-H α-subunit RNA

Translation of rat α-subunit RNA in a rabbit reticulocyte lysate system containing [^{35}S]methionine produced several radiolabelled products (Figure 1), including the expected ~60 kDa full-length polypeptide (lane 4). The presence of lower molecular weight polypeptides was probably due to translation initiation at internal methionine codons (Kozak, 1990). Processing of the full-length polypeptide product was effected by addition of canine pancreatic microsomal membranes to the translation and resulted in the production of two extra polypeptides of molecular weight greater than the 60 kDa band present in the translations without microsomal vesicles (compare lanes 5 and 4). Since glycosylation increases the apparent molecular weight of proteins on SDS–PAGE, and since the α-subunit has two potential glycosylation sites (I.Hopkinson, S.A.Smith, M.E.Grant and J.Rosamond, unpublished sequence data), these two bands were inferred to be glycosylated forms of the α-subunit. This was confirmed by digestion of the translation product (lane 11) with a deglycosylating enzyme endoglycosidase H (EndoH). EndoH treatment produced a single polypeptide (lane 12) of mobility similar to that of the lowest band of the three present in the control sample (lane 11). Thus, the two polypeptides sensitive to EndoH treatment corresponded to translocated, glycosylated α-subunit.

Identification of translocated polypeptides was achieved by proteinase K digestion of translation products—proteins translocated into the lumen of the microsomal vesicles are protected from protease degradation unless a detergent is added to solubilize the vesicles. Comparison of proteinase K treatment of translations carried out in the absence (lane 6) or presence (lane 8) of microsomal vesicles, showed that all translation products were degraded in the former, whereas three major products were protected from degradation in the latter. Solubilization of microsomal vesicles with Triton X-100 followed by proteinase K treatment resulted in the loss of these protected bands (compare lanes 8 and 9). It is appropriate to note that the lower molecular weight products (<60 kDa) observed in untreated translation samples (e.g. lanes 4 and 5), were degraded by proteinase K even in the presence of microsomal membranes (lane 8) and, thus, were not translocated polypeptides. Purification of the microsomal vesicles from translations by centrifugation through a sucrose cushion removed all non-translocated polypeptides and subsequent analysis by SDS–PAGE (lane 11) revealed a similar band pattern to that of proteinase K-treated translation products synthesized in the presence of microsomal vesicles (lane 8).

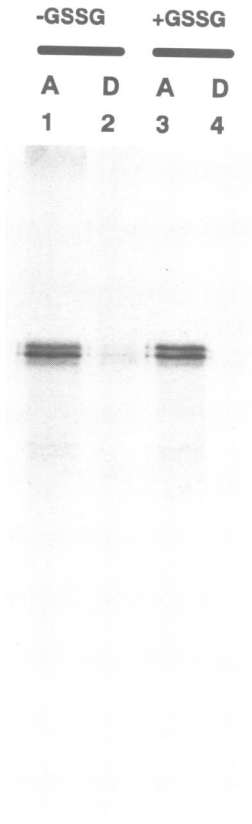

Fig. 3. Membrane association of translocation products: Triton X-114 phase separation. Microsomes were isolated from translation of P4-H α-subunit carried out in the presence of microsomes and the absence (lanes 1 and 2) or the presence of GSSG (lanes 3 and 4). Purified microsomes were subjected to a Triton X-114 phase separation procedure to yield a detergent-poor phase (A) and a detergent-rich phase (D). Products of translation were separated by SDS−PAGE. The gel was fixed, soaked in Amplify, dried and developed by fluorography.

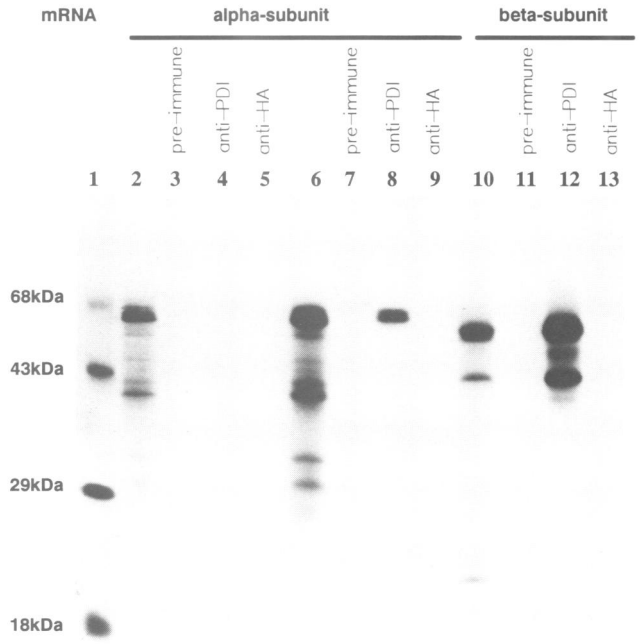

Fig. 4. Immunoprecipitation of P4-H α-subunit with antibodies to PDI. P4-H α- and β-subunit mRNAs were translated in the presence (lanes 6−9) or absence (lanes 2−5 and 10−13) of GSSG. Translation products were immunoprecipitated from 25 μl translation mixture using pre-immune or polyclonal rabbit antisera as indicated. Products of translation were separated by SDS−PAGE. The gel was fixed, soaked in Amplify, dried and developed by fluorography. Lane 1 contained ^{14}C-labelled protein standards of the sizes indicated and lanes 2, 6 and 10 contained aliquots (1 μl) of the translation mixture prior to immunoprecipitation.

Membrane association of translation products

The α-subunit of P4-H was translated in the presence of microsomal vesicles which contain the β-subunit (PDI) to mimic the conditions for P4-H assembly *in vivo* (Berg *et al.*, 1980). Glutathione disulfide (GSSG), an oxidant, was added to translations to enable the formation of native disulfide bonds in the nascent polypeptides—the translation system is a reducing environment (2 mM DTT), and addition of GSSG (3 mM) alters the redox potential of the system in favour of native disulfide bond formation (Scheele and Jacoby, 1982). To determine the localization of the newly synthesized translocated α-subunit, we utilized two techniques. The first involved disruption of microsomes by incubation in sodium carbonate (0.1 M, pH 11.5) which converts closed vesicles to open membrane sheets (Fujiki *et al.*, 1982). Subsequent centrifugation of such samples yields a supernatant containing lumenal proteins and a pellet containing membrane-bound/associated proteins. SDS−PAGE analysis of microsomal protein fractions (Figure 2) showed that for control translations of γ-interferon (a lumenal hydrophilic protein) and influenza virus haemagglutinin (a protein containing a transmembrane domain), carried out in the absence of GSSG, the synthesized proteins were recovered in the predicted lumenal or membranous fractions (lanes 1−3 and 10−12, respectively). Similar results were obtained when the translations were carried out in the presence of GSSG (results not shown). When the same analysis was carried out with the α-subunit the result was dependent on whether GSSG was present during the translation. Thus, in the absence of GSSG the translation product was found predominantly in the pellet (lanes 4−6) whereas in the presence of GSSG the protein was in the supernatant (lane 7−9). The presence of α-subunit in the pellet when translations were carried out in the absence of GSSG was unexpected since there is no evidence to suggest that P4-H is membrane bound. However, it is known that isolated α-subunits aggregate and become insoluble when dissociated from the β-subunit (Tuderman *et al.*, 1975). These results indicate that in the presence of GSSG the protein remains soluble, possibly due to its association with PDI, and appears in the supernatant fraction whereas in the absence of GSSG the translated α-subunit may become aggregated and therefore appear in the pellet on centrifugation.

The second technique employed to determine the submicrosomal location of translated α-subunit was a modified form (Tiruppathi *et al.*, 1986) of the Triton X-114 phase separation technique (Bordier, 1981). In this procedure membrane-bound and hydrophobic proteins appear in the detergent-rich phase and hydrophilic or non-membrane-bound proteins appear in the detergent-poor (aqueous) phase. The results shown in Figure 3 indicate that α-subunit translated in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of GSSG appears only in the aqueous phase. This indicates that the protein is not membrane associated and

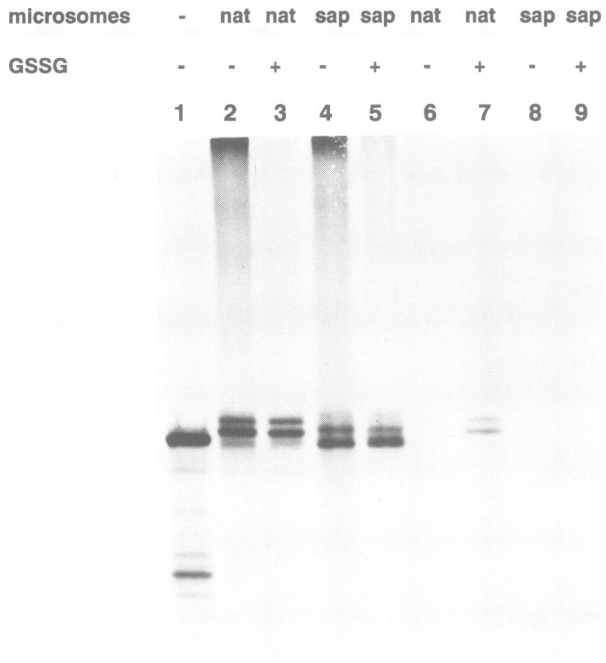

Fig. 5. Effect of PDI depletion on the immunoprecipitation of α-subunit with PDI antibody. Translations of α-subunit RNA were carried out in the presence or absence of native (nat) or saponin washed (sap) microsomes, and in the presence or absence of GSSG as indicated. Products of translation were separated by SDS−PAGE through a 10% acrylamide gel. The gel was fixed, dried and developed by autoradiography. Lane 1 contains an aliquot of α-subunit translated in the absence of microsomes and shows the position of non-glycosylated α-subunit. Lanes 2−5 contain aliquots of sucrose cushion purified microsomes isolated from the translations indicated. Lanes 6−9 show the immunoprecipitates derived from immunoprecipitation of samples in lanes 2−5, respectively, using polyclonal antiserum directed against PDI.

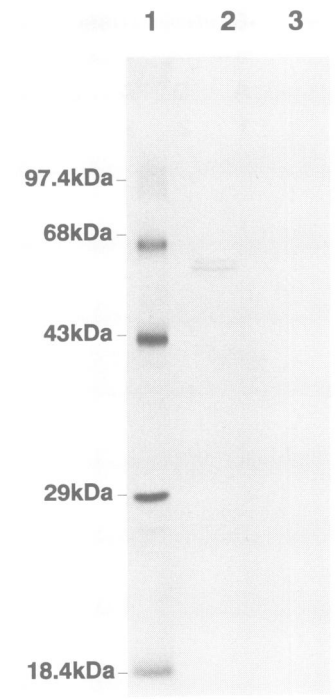

Fig. 6. Immunoprecipitation of α-subunit with anti-BiP antibodies. Translations of α-subunit RNA were carried out in the presence or absence of native microsomes, and in the presence or absence of GSSG as indicated. Products of translation were separated by SDS−PAGE through a 10% acrylamide gel. The gel was fixed, dried and developed by autoradiography. Lane 1: molecular weight markers. Lane 2: translation carried out in the absence of GSSG and immunoprecipitated with anti-BiP antibodies after post-translational depletion of ATP with apyrase. Lane 3: as lane 2 but translations carried out in the presence of GSSG.

therefore confirms that the appearance of the α-subunit in the pellet fraction after carbonate washing reflects the formation of aggregates.

Immunoprecipitation of α-subunit with antibodies to β-subunit (PDI)

The formation of a complex between newly synthesized α-subunits and endogenous β-subunits was examined by immunoprecipitation of the products of translation using polyclonal antibodies raised against PDI (P4-H β-subunit). This antibody was capable of precipitating translation product synthesized from RNA coding for the β-subunit of human P4-H (Figure 4, lane 12). The results show that α-subunit was immunoprecipitated from translations where GSSG was present (Figure 4, lane 8; Figure 5, lane 7) but not from those where GSSG was absent (Figure 4, lane 4; Figure 5, lane 6). The precipitation of the α-subunit with antibodies against PDI was not due to non-specific binding as neither pre-immune serum nor antibodies to an unrelated protein, influenza virus haemagglutinin, could precipitate this protein (lanes 7 and 9). The ability of antibodies against the β-subunit to precipitate translated, translocated α-subunit strongly suggests that a complex has formed between these two proteins.

Further evidence to support this conclusion was provided when the α-subunit was translated in the presence of PDI-depleted microsomal vesicles (Figure 5). For this experiment microsomes were purified from the translation mixture to ensure that equal amounts of translocated α-subunit were present during the immunoprecipitation. The saponin-wash treatment did not prevent translocation of the α-subunit but did have an effect on glycosylation (lanes 4 and 5). Thus, less of the translocated polypeptide was glycosylated and the glycosylated product that was formed had a slightly higher molecular weight than the glycoprotein synthesized in the presence of untreated microsomes (compare lanes 3 and 4). As described previously (Bulleid and Freedman, 1990), the latter observation is probably due to the removal of glucosidases during the washing procedure, thus preventing the removal of terminal glucose residues from the oligosaccharide side-chains. As expected, removal of PDI and other soluble ER proteins from microsomes prevented the precipitation of translocated α-subunit with the PDI antibody even when GSSG was present during the translation (lanes 8 and 9). This demonstrates that the precipitation of α-subunit with PDI antibody was not due to non-specific interaction of the antibody with the α-subunit in the presence of GSSG. The α-subunit synthesized in the presence of saponin-washed microsomes was also shown to have aggregated by the appearance of the protein in the pellet after carbonate washing, even when GSSG was present during translation (results not shown). This indicates that for this protein to remain soluble it must associate with either the β-subunit, which it has been shown to do, or with another ER soluble protein or proteins, and that the presence of GSSG during the translation is not the only requirement for correct folding of the α-subunit.

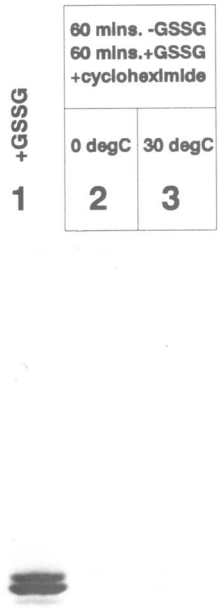

Fig. 7. Effect of post-translational addition of GSSG on the yield of immunoprecipitable α-subunit. Products of translation were separated by SDS−PAGE. The gel was fixed, dried and developed by autoradiography. The α-subunit RNA was translated in the presence of microsomes (lanes 1−3) and in the presence (lane 1) or absence (lanes 2 and 3) of GSSG. Following termination of translation by the addition of cycloheximide, GSSG was added (lanes 2 and 3) and the reactions incubated for a further 60 min, at 0 and 30°C as indicated. All samples were then immunoprecipitated using polyclonal antiserum directed against PDI.

To determine whether the α-subunit associates with BiP translations carried out in either the presence or absence of GSSG were first depleted of ATP post-translationally and then immunoprecipitated with antibodies to BiP. As can be seen (Figure 6), when translation was carried out in the presence of GSSG the synthesized α-subunit was not precipitated; however, α-subunit synthesized in the absence of GSSG was precipitated with the BiP antibody. This result indicates that this BiP is associated with the α-subunit aggregates.

To investigate the requirement for GSSG during translation, GSSG was added post-translationally to translation reactions carried out in the absence of GSSG after protein synthesis had been terminated by the addition of cycloheximide. The incubation was continued either on ice or at 30°C for a further hour before immunoprecipitation with antibodies to PDI. Compared with the control translation with GSSG (Figure 7, lane 1), post-translational addition of GSSG followed by incubation on ice (lane 2) or at 30°C (lane 3) yielded very low amounts of immunoprecipitable α-subunit. These results indicate that the formation of the

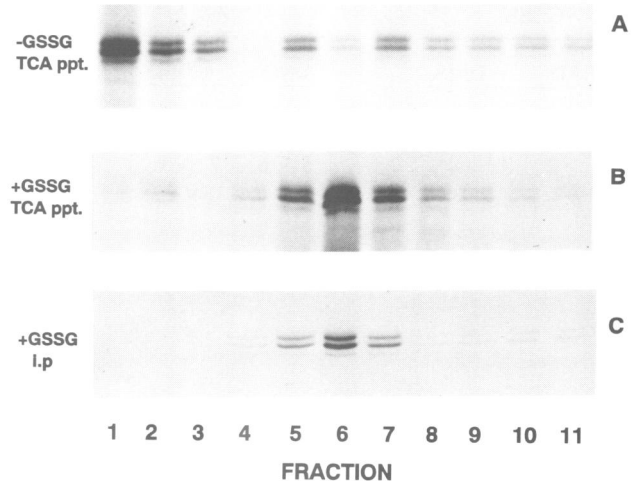

Fig. 8. Sucrose gradient size fractionation of translation products. Microsomes were isolated from translations of α-subunit RNA carried out in the presence or absence of GSSG as indicated. Microsomal samples were resuspended and fractionated on a 5−25% sucrose gradient. Samples were separated by SDS−PAGE through a 10% polyacrylamide gel. The gel was fixed, dried and developed by autoradiography. Fractions were either precipitated with TCA (gels A and B) or immunoprecipitated using polyclonal serum directed against PDI (gel C). Fractions 1 and 11 represent the bottom and the top of the gradients, respectively.

complex occurs co-translationally or very soon after translation has been completed, and that GSSG must be present as the polypeptide is being translocated for complex formation to occur.

Size fractionation of translation products

To characterize the complex formed between the translocated α-subunit and endogenous β-subunit we carried out sucrose gradient fractionation of the translation products. Microsomal vesicles were isolated from translations and subjected to centrifugation through 5−25% sucrose gradients containing the non-ionic detergent lauryl maltoside (Segal *et al.*, 1992). Gradients were fractionated and either immunoprecipitated using PDI antibody or precipitated with trichloroacetic acid and analysed by SDS−PAGE. The majority of the α-subunit synthesized in the absence of GSSG sedimented at the bottom of the gradient (Figure 8A). However, α-subunit synthesized in the presence of GSSG sedimented only in fractions towards the middle of the gradient (Figure 8B). When the fractions were immunoprecipitated no product was precipitated from translations carried out in the absence of GSSG (data not shown) but the α-subunit translated in the presence of GSSG was precipitated (Figure 8C). When purified P4-H was sedimented on a similar gradient the sedimentation pattern was identical to that found with the translation product synthesized in the presence of GSSG (data not shown). These results indicate that in the absence of GSSG, the majority of the translocated α-subunit forms aggregates which are sedimented at the bottom of these gradients whereas in the presence of GSSG the α-subunit remains soluble and forms a complex with the β-subunit.

Cross-linking of translation products

The homobifunctional amine-reactive reagent dimethylsuberimidate (DMS) was used to cross-link lysyl residues of the α−β complex formed during the cell-free translation of the α-subunit. The procedure used was as

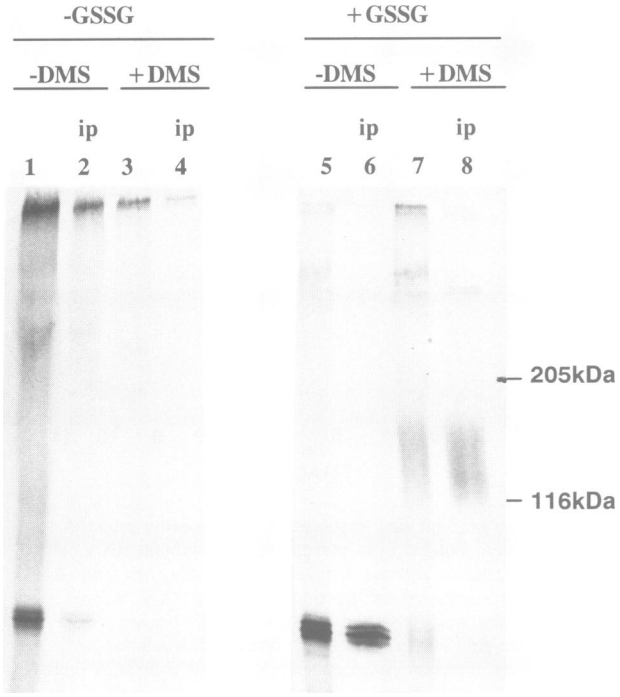

Fig. 9. Cross-linking of products of translation. Microsomal vesicles isolated from 25 μl of α-subunit translation (carried out in the presence or absence of GSSG as indicated) were resuspended in 10 μl LM buffer and incubated with the cross-linking reagents DMS (+DMS) or buffer (−DMS) in a final volume of 50 μl. An aliquot (5 μl) of the reaction mixture was removed for analysis by SDS−PAGE (lanes 1, 3, 5 and 7), the remainder of the sample (45 μl) was immunoprecipitated with polyclonal rabbit antiserum against PDI and the immunoprecipitates were analysed by SDS−PAGE (lanes 2, 4, 6 and 8) under reducing conditions through a 5−10% SDS−polyacrylamide gradient gel. The positions of unlabelled size markers β-galactosidase (116 kDa) and myosin (205 kDa) are shown.

described by Swaney and O'Brien (1978) but the duration of incubation was increased in order to produce significant product yields. Analysis of cross-linking reaction products on 5−10% SDS−PAGE gradients (Figure 9) enabled comparison of monomeric and cross-linked species. Cross-linking of α-subunit translated in the presence of GSSG yielded a 120−150 kDa smear and a second fainter band at ~300 kDa (lane 7); no such products were obtained from translations carried out in the absence of GSSG (lane 3). In addition, the cross-linked products (lane 7) were immunoprecipitable using antibodies against PDI (lane 8). Thus the species present at 120−150 kDa probably corresponds to a αβ dimer whereas the higher molecular weight band corresponds to the tetramer. The relative molecular weights of these complexes are greater than those calculated from the individual subunit molecular weights. Thus, a dimer between these two proteins would have an expected relative molecular weight of 120 kDa and a tetramer 240 kDa. It is important to note, however, that modification of many of the lysyl residues present on each subunit may occur during the cross-linking procedures, increasing the apparent molecular weight of the cross-linked species, and that cross-linked polypeptides will not be linear chains and would, therefore, not be expected to migrate on SDS−PAGE at the appropriate molecular weight.

Discussion

The study of the interactions between the subunits of purified P4-H has been hampered by the strong tendency of the α-subunit to form insoluble aggregates when dissociated from the β-subunit (Tuderman *et al.*, 1975; Berg *et al.*, 1977). To date no details of the interactions between the subunits in the P4-H tetramer have been elucidated, although many studies on the enzymic reaction, its substrates, co-factors and inhibitors and the molecular biology of the enzyme have been carried out (recently reviewed by Kivirikko *et al.*, 1992). In an attempt to obtain information about the subunit interactions and the assembly of P4-H we have studied the cell-free synthesis of the α-subunit and its assembly with endogenous microsomal β-subunit (PDI).

The α-subunit translated in this system was translocated into microsomal vesicles and glycosylated, resulting in the production of two differentially glycosylated forms. Rat α-subunit has two potential glycosylation sites as do the α-subunits from chick (Bassuk *et al.*, 1989) and human P4-H (Helaakoski *et al.*, 1989); glycosylation on either one or both of these sites may give rise to the different glycoforms. P4-H isolated from chick embryo fibroblasts (Kedersha *et al.*, 1985a) and human skin (Kuuti *et al.*, 1975) contains two forms of the α-subunit that have been proposed by Kedersha *et al.* (1985a,b) to arise from two highly homologous gene products with slight differences in amino acid composition which result in the glycosylation at exclusively one or both of the glycosylation sites. There is also evidence for alternative splicing of the human α-subunit mRNA, and although the sequences involved include neither of the potential glycosylation sites (Helaakoski *et al.*, 1989), it is possible that this may account for the appearance of two different α-subunit glycoforms. However, it is clear that in the work presented here the α-subunit is derived from only one type of RNA transcript and thus, alternative splicing cannot be involved in the production of the two α-subunit glycoforms synthesized in our cell-free system. This demonstrates that different glycoforms of the α-subunit can be generated from a single RNA transcript.

The main aim of this study was to assemble the $\alpha_2\beta_2$ tetramer of P4-H. To establish the conditions necessary for the formation of a complex between newly synthesized α-subunit and endogenous β-subunit, we first had to consider whether there was a requirement for the formation of disulfide bonds. Purified P4-H tetramers can be dissociated by the addition of DTT to 1 mM (Prockop *et al.*, 1976; Nietfeld *et al.*, 1981) suggesting that disulfide bonds are necessary to maintain the complex. However, it is unlikely that there are disulfide bonds between the subunits since dissociation can also be effected by addition of urea (Nietfeld *et al.*, 1981) or alkylation in the absence of reduction (Berg and Prockop, 1973). It is probable, therefore, that there is at least one intramolecular disulfide bond in the α- or β-subunit which on reduction leads to dissociation of the $\alpha_2\beta_2$ tetramer. The β-subunit possesses six cysteine residues, four of which are present in the two active-site disulfides required for PDI activity, the remaining two having no defined role (Hawkins and Freedman, 1991). The two pairs of active-site disulfides are not likely to be involved in the α−β subunit interaction since (i) the β-subunit when part of the P4-H tetramer retains half of its PDI activity (Koivu *et al.*,

1987) and (ii) in co-transfection studies using a baculovirus expression system, removal of the cysteines at either one or both of the active-sites of PDI had no effect on the assembly of the mutant β-subunits with co-expressed α-subunits or on the resulting P4-H activity (Vuori *et al.*, 1992). The α-subunit has five cysteine residues, some of which are probably involved in coordination of Fe(II) in the active-site; the remainder may be involved in intramolecular disulfide bonds (Kivirikko *et al.*, 1992). There is as yet no evidence for the existence of intramolecular disulfide bonds in the α-subunit. To investigate the association of α-subunit with the β-subunit, we attempted to precipitate this protein with antibodies raised against PDI. The results clearly indicate that a complex between the subunits was formed but only when GSSG was present during the translation. GSSG alters the redox conditions such that disulfide bonds may form in newly synthesized proteins (Scheele and Jaçoby, 1982). The fact that the addition of GSSG is a requirement for the formation of the complex between these two proteins suggests that the formation of a disulfide bond or bonds is necessary for these proteins to associate.

To determine whether intramolecular disulfides were formed within the α-subunit, we compared the mobility of the translocated α-subunit separated under reducing or non-reducing conditions (D.C.A.John and N.J.Bulleid, unpublished data). No difference in mobility was observed, indicating that either no disulfide bonds were formed or that if disulfide bonds had formed then there was no significant decrease in the hydrodynamic volume of the polypeptide. However, the presence of GSSG during the translation did have a marked effect on the folding of the protein. Thus, in the absence of GSSG the α-subunit aggregated as judged by its appearance in the pellet fraction after carbonate washing and its sedimentation at the bottom of sucrose gradients after centrifugation. However, in the presence of GSSG it remained soluble, indicating that the protein had folded correctly. As GSSG is unlikely to affect the folding of the protein other than by facilitating disulfide bond formation we therefore conclude that an intra-molecular disulfide bond or bonds must form within the α-subunit to ensure correct folding of this protein prior to its association with the β-subunit.

That the assembly of the $\alpha-\beta$ complex occurred either co-translationally or very soon after the α-subunit was translocated into the lumen of the microsomal vesicle, was demonstrated by the fact that when GSSG was added post-translationally no complex formed, probably due to the irreversible aggregation of the α-subunit. Time courses of the appearance of translocated α-subunit and complex formation also demonstrated that the $\alpha-\beta$ complex formed very soon after translocation of the α-subunit (D.C.A.John and N.J.Bulleid, unpublished results). These results indicate that PDI plays a crucial role in preventing aggregation of the α-subunit, a tendency which the α-subunit isolated from P4-H exhibits strongly (Tuderman *et al.*, 1975). This is consistent with the proposed role of PDI as part of the microsomal triglyceride transfer protein complex (Wetterau *et al.*, 1991). We have also shown that the ER lumenal protein BiP is associated with α-subunit aggregates. Such an association of BiP with proteins synthesized and translocated *in vitro* has been described previously for proteins that are incorrectly disulfide bonded or aberrantly glycosylated (Kassenbrock *et al.*, 1988) or membrane proteins that are aggregated (Yilla *et al.*, 1992). We did not detect any association of BiP with α-subunit synthesized in the presence of GSSG but this does not rule out the possibility that BiP may transiently interact with this protein prior to its assembly with the β-subunit.

In our system we report the formation of a complex between the α- and β-subunits of P4-H which can be chemically cross-linked to form predominantly an αβ dimer with a lower yield of tetramer. The dimer may represent a stable intermediate in the assembly of the tetrameric enzyme which requires a further period of maturation before forming the fully assembled enzyme. Alternatively the conditions used during the solubilization of the microsomal membranes and analysis of the complex may have caused the dissociation of any tetramers that may have formed. Indeed purified P4-H had an identical sedimentation pattern on sucrose gradients to the complex formed *in vitro*. Attempts to characterize the complex by size exclusion chromatography were unsuccessful as radiolabelled α-subunit was detected in all column fractions probably due to interaction of the aggregated protein with the column matrix (D.C.A.John and N.J.Bulleid, unpublished results).

This study shows for the first time that P4-H may be assembled in a cell-free system from newly synthesized α-subunit and a pre-existing pool of β-subunits. We now have an experimental system whereby the effect of mutations within the α-subunit on the assembly of P4-H can be determined. The multifunctional protein PDI may be performing a role in maintaining the solubility of otherwise insoluble subunits such as the α-subunit of P4-H and the 88 kDa subunit of the microsomal triglyceride transfer subunit. Other ER lumenal proteins such as BiP have been shown to interact with newly synthesized proteins and prevent their aggregation (Segal *et al.*, 1992). However, in these cases the interaction is usually transient, preventing the aggregation of unfolded monomers, or more permanent where the association is with preformed aggregates (Blount and Merlie, 1991; Braakman *et al.*, 1992; Knittler and Haas, 1992). This is in contrast to the stable interaction of PDI with these proteins where PDI remains part of the assembled functional enzyme complex.

Materials and methods

Restriction enzymes, endoglycosidase H, nucleotides and lauryl maltoside were purchased from Boehringer-Mannheim (Lewes, UK). Nuclease treated rabbit reticulocyte lysate, T3 and T7 RNA polymerases and RNAsin ribonuclease inhibitor were purchased from Promega (Southampton, UK). The phagemid vector pBluescript was purchased from Stratagene (Cambridge, UK). L-[^{35}S]methionine (>1000 Ci/mmol) and Amplify were purchased from Amersham International (Bucks, UK). Cycloheximide, dimethylsuberimidate and protein A–Sepharose were from Sigma (Poole, Dorset, UK). ^{14}C-labelled protein molecular weight standards were prepared by reductive methylation of lysyl residues using the procedure of Dottavio-Martin and Ravel (1978). Bovine PDI was prepared by the method of Lambert and Freedman (1983) and used to raise polyclonal antiserum in rabbits. The purified chick prolyl 4-hydroxylase was a gift from Dr T.J.Franklin, ICI Pharmaceuticals, Alderley Park, UK. The cDNA clone coding for influenza virus haemagglutinin (HA) from A/Japan/305/57 strain in pGEM-4Z and the polyclonal rabbit antiserum directed against HA were gifts from Dr M.J.Gething, Howard Hughes Medical Institute, Dallas, Texas. The monoclonal antibody to BiP was a gift from Dr Linda Hendershot, St Jude Children's Research Hospital, Memphis, TN 38101. The construction of pUK1013 which contains the human γ-interferon cDNA has been

described previously (Bulleid *et al.*, 1990). The plasmid pUK1006 containing the human PDI cDNA was constructed in pBluescript from cDNA clones provided by Dr Taina Pihlajaniemi, University of Oulu, Finland (Pihlajaniemi *et al.*, 1987). A 2.0 kb *Eco*RI fragment containing the cDNA of the rat P4-H α-subunit was isolated from a neonatal rat aorta cDNA library (I.Hopkinson, S.A.Smith, M.E.Grant and J.Rosamond, unpublished results) and subcloned into the *Eco*RI site of pBluescript vector—plasmids pDJ1 and pDJ2 contain the inserted cDNA sequence oriented for transcription by either T7 or T3 RNA polymerase, respectively.

Microsomal vesicles were prepared from canine pancreas as described previously (Austen *et al.*, 1984). Microsomal vesicles were depleted of PDI by treatment with saponin (1% w/v) as described previously (Bulleid and Freedman, 1990).

Cell-free transcription and translation

Transcriptions were carried out essentially as described by Gurevich *et al.* (1991). Linearized DNA (5 μg) was incubated for 2 h at 37°C in 50 μl of 80 mM HEPES–KOH, pH 7.5, containing 12 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, 3 mM each of ATP, CTP, GTP and UTP, 200 U/ml RNAsin and 1500 U/ml T3 or T7 RNA polymerase. RNA was resuspended in RNase-free water (50 μl) containing 1 mM DTT and 200 U/ml RNAsin.

Translation of RNA transcripts was carried out in a micrococcal nuclease-treated rabbit reticulocyte lysate system supplemented with canine pancreatic microsomal membranes. Translation mixtures (25 μl) contained 18 μl rabbit reticulocyte lysate, 20 μM each of 19 amino acids (minus methionine), 15 μCi [^{35}S]methionine and 0.5 μg RNA transcript. Canine pancreatic microsomes (4.8 A280 units/ml) and GSSG (3 mM) were added where required as the final components of the translation mixture. Translations (60 min, 30°C) were terminated by incubation on ice or by the addition of cycloheximide (2 mM) where appropriate.

Post-translational proteolysis, isolation of microsomes, deglycosylation and immunoprecipitation

Translation mixtures were assayed for translocation by treating post-translationally with proteinase K as described previously (Bulleid and Freedman, 1988). Microsomal vesicles were isolated from translation mixtures by centrifugation (150 000 *g*, 10 min, 4°C) through a sucrose cushion (50 mM HEPES–KOH, ph 7.5, 100 mM KCl and 0.5 M sucrose). The pellet was resuspended in 50 mM HEPES–KOH, pH 7.5, 100 mM KCl, 0.25 mM sucrose and this sample was spun through a sucrose cushion as above. The final pellet was resuspended in a buffer appropriate to the subsequent procedure. Products of translation were assayed for glycosylation by treatment with endoglycosidase H as described previously (Bulleid *et al.*, 1992).

Immunoprecipitation of translation products was carried out in 500 μl buffer A [50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, 0.25% (w/v) gelatin, 0.05% (w/v) Nonidet P40 and 0.02% sodium azide]. For immunoprecipitations with anti-BiP, translation mixtures were depleted of ATP by the addition of apyrase (100 U/ml) for 2 min at 37°C prior to addition of buffer A. Samples were precleared with 40 μl protein A–Sepharose (10% v/v suspension) and immunoprecipitated overnight at 4°C with 1 μl of the appropriate polyclonal antiserum or 5 μl of the BiP monoclonal antibody. The samples were then incubated for 60 min at 4°C with 40 μl protein A–Sepharose suspension before recovery of the immunoprecipitates by centrifugation. Pelleted Sepharose beads were washed three times in 1 ml buffer A and resuspended in SDS–PAGE sample buffer.

Sodium carbonate treatment and Triton X-114 phase separation

Microsomes isolated from translations by centrifugation through sucrose cushions were subjected to sodium carbonate treatment by a method adapted from that of Fujiki *et al.* (1982). Briefly, isolated microsomes were resuspended in 300 μl ice-cold sodium carbonate (0.1 M, pH 11.5) and incubated on ice for 10 min followed by centrifugation (200 000 *g*, 60 min) to recover pellet and supernatant fractions. This procedure was repeated twice more on the pellet fraction and the supernatants of the three spins were pooled and precipitated with 10% (w/v) TCA. Pellets and supernatant TCA precipitates were then resuspended in equal volumes of SDS–PAGE loading buffer.

Triton X-114 phase separation treatment of microsomes isolated from translations was carried out essentially as described by Tiruppathi *et al.* (1986) modified from that of Bordier (1981).

Sucrose gradient analysis

Microsomes were isolated from translation mixtures by centrifugation through sucrose cushions and resuspended in 0.5 ml LM buffer (50 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 6 mM lauryl maltoside and 1 mM PMSF; Segal *et al.*, 1992). This sample was loaded on to a 5–25% sucrose gradient prepared in LM buffer and subjected to centrifugation for 17 h in an SW40 rotor (40 000 r.p.m., 4°C). Gradient fractions were collected and proteins immunoprecipitated and/or TCA precipitated before analysis by SDS–PAGE under reducing conditions.

Chemical cross-linking of translation products

The DMS was used to cross-link putative P4-H α–β subunit complexes present in microsomes isolated from α-subunit translations. Briefly, microsomes isolated from 25 μl translation reaction were resuspended in 10 μl LM buffer (minus PMSF) to which was added 35 μl water and 5 μl DMS (60 mg/ml in 1 M triethanolamine, pH 9.7). Control incubation was prepared as above except 5 μl 1 M triethanolamine was added in place of the DMS solution. Samples were incubated at 25°C for 20 h. Reactions were terminated by freezing and/or addition of SDS–PAGE sample buffer prior to electrophoresis in a 5–10% SDS–polyacrylamide gradient gel.

SDS–PAGE

Samples were prepared for electrophoresis by mixing with 5 vol SDS–PAGE sample buffer [60 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue], dithiothreitol was then added (50 mM) and the samples were boiled for 5 min. Electrophoresis through 12.5% gels (unless otherwise stated) was carried out in the presence of SDS by the method of Laemmli (1970). Gels were subjected to autoradiography at room temperature or fluorography at −80°C after incubation in Amplify.

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